Effects of surface-treated cpTi and Ti6Al4V alloy on the initial attachment of human osteoblast cells

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This study concerns the effect of simple surface treatments on the nature of the oxide layer, of commercially pure titanium (cpTi) and Ti6Al4V alloy substrates and their effect on human osteoblast cells (HOBS). After treatment the surfaces were analyzed by X-ray photoelectron spectroscopy (XPS) in order to identify the surface groups responsible for the cell attachment process. The assessment of cell attachment was monitored by the Alamar blue assay (AB), measuring cell activity, in three types of media: phosphate-buffered saline (PBS), serum containing and serum-free Dulbecco's modified Eagle's cell culture medium (SER + and SERF respectively). XPS analysis of the treated surfaces revealed consistent peaks representative of TiO_2 on all surfaces and Ti^0 and Ti_2O_3 on the non-heat-treated surfaces. The cell activity assays indicated that there were no significant differences in cellular activity caused by surface treatments, but the cellular activity compared between the three types of medium was greatest in the PBS over the initial stages of attachment.

1. Introduction

Titanium is a widely used biomaterial in both dental and orthopaedic fields [1–3]. Its biocompatibility is attributed to the overlaying oxide layer and its hydrated state [4]. The nature of the oxide layers and their effect on biocompatibility have become an important area of research [5–7]. It has been shown that variation in the preparation of titanium and its alloys has an effect on the dissolution behavior of these materials in biological environments [8–10]. Biological activity on material surfaces is dependent on a combination of the cell interactions with the material surface and biological fluid as well as material interactions with the biological surroundings [11]. This study investigates the effect of simple surface treatments to produce different oxide layers. These include nitric acid passivation, and heat treatments at 400 °C of both cpTi and Ti6Al4V substrates. In addition the influence of these surfaces on cell attachment and activity and the nature of the culture media on them has been studied.

2. Methods and materials

2.1. Sample preparation

cpTi (Industrial Metal Industries, Titanium 155) and Ti6Al4V sheet (made to BS7252 Part 3) were machined into 15 × 1 mm discs for XPS and 6 × 1 mm discs for cell culture tests. The samples were mechanically polished to a 0.06 μm finish, using colloidal silica gel. They were then cleaned in an ultrasonic bath with 5% Decon 90 solution. The samples were surface treated in the following manner: (a) untreated (cpTi/Ti64), (b) heated at 400 °C for 45 min (cp400/64400) or (c) immersed in a 30% nitric acid bath for 10 min (cpN10/64N10). Tissue culture plastic was used as a control in cell culture tests (TCP).

2.2. Surface analysis

X-ray photoelectron spectroscopy (XPS) data was obtained in an ESCALAB 5 Mk II, equipped with a monochromatic AlK_α X-ray source (1486.6 eV). All spectra were referenced by setting the carbon C1s to 285.0 eV. The survey spectra were collected over a binding energy range of 0–1000 eV at a take-off angle of 45°, and pass energy of 50 eV. High-resolution spectra were taken over a range of 15–20 eV, at a 45° take-off angle with pass energy of 20 eV. The samples were prepared in triplicate.

2.3. Cell culturing

Primary human osteoblasts, derived from femoral head trabecular bone were incubated in (a) Dulbecco's modified Eagle's complete cell culture media (SER +), (b) serum-free cell culture media (SERF) and (c) phosphate-buffered saline (PBS) at 37 °C, 5% CO_2 for 90 min. The samples were prepared in triplicate, and into

each well of a 96-well plate a dilution of 10 000 cells/100 μl of media was placed. The Alamar blue (AB) assay was used to determine the cell activity. The AB assay is able to follow the activity of cells, due to the incorporation of an oxidation/reduction (Redox) indicator in solution that both fluoresces and changes color in response to the growth of cells. Analysis of variance (ANOVA) tests were also performed for the identification of significant differences in the levels of cellular activity between experimental groups.

3. Results and discussion

3.1. XPS results

Spectra were obtained on the cpTi and Ti6Al4V alloy samples which had undergone the three surface treatments. Titanium, oxygen and carbon peaks were observed on all the cpTi and Ti6Al4V alloy samples. Aluminum was also observed on the Ti6Al4V alloy samples. At no time was vanadium observed on the treated surfaces. The heat-treated samples, in addition, showed the presence of trace amounts of calcium, for both cpTi and Ti6Al4V alloy samples. Table I summarizes the total surface composition in atomic percentages. The presence of both carbon and calcium are due to atmospheric surface contamination, which could have been as a result of contamination from the mechanical polishing or heat treatment environments or from the solvents used to clean the specimens prior to testing.

High-resolution spectra of the C1s peaks indicated that it was made up of three peaks. The main peak with binding energy of 285 eV is related to the C–C organic bonds in saturated hydrocarbon groups. The other two peaks with binding energies approximately 1.5 eV and 3.5 eV higher than the peak at 285 eV correspond to C–O and C═O species, respectively [12].

A selection of characteristic high-resolution Ti2p spectra resulting from each surface treatment can be seen in Fig. 1a–c. A comparison of the untreated and nitric acid passivated samples shows that similar results were obtained. The peaks indicate that TiO_2 (Ti^{4+}) is the major surface component for both cpTi and Ti6Al4V alloy samples. The presence of the suboxide Ti_2O_3 (Ti^{3+}) and elemental titanium metal (Ti^0) were also seen at the surface. In the heat-treated samples for both cpTi and Ti6Al4V alloy, the Ti2p peak regions showed only the presence of TiO_2 on the surface. The peak positions for TiO_2 were consistent with binding energy shifts expected for titanium metal in combination with oxygen in the form of TiO_2 [13]. The binding energy values of Ti^{4+} 2p3/2 and Ti^{4+} 2p1/2 were between 458.6 and 458.9 eV and 464.2 and 464.6 eV, respectively. The Ti^0 2p3/2 and Ti^0 2p1/2 binding energies occur at 454.0–454.6 eV and 461.5–461.7 eV, respectively. The binding energy of the Ti_2O_3 peaks occur at 456.0–456.9 eV. Table II summarizes the surface chemistry in atomic percentages, considering only the Ti2p region. Since both carbon and to a lesser extent oxygen, contribute to the contamination layer, it is necessary that the true effect of surface treatment on the titanium chemistry be ascertained.

TABLE I Total surface composition in atomic percentages for the treated surfaces

At %	Ti	O	C	Al
cpTi	23.28	55.83	20.89	
cpN10	23.09	54.57	22.35	
cp400	21.92	62.27	15.81	
Ti64	17.63	51.38	28.38	2.61
64N10	19.25	53.45	26.02	1.28
64400	17.46	55.58	22.86	4.10

The high-resolution A12p spectra results for the Ti6A14V alloy samples are shown in Fig. 2a and b. It can be seen that all sample surface treatments result in the formation of aluminum oxide (Al_2O_3), at binding energies ranging from 73.3–74.3 eV. The untreated, as-polished samples were the only surfaces to indicate the presence of elemental aluminum metal (Al^0) at binding energies ranging from 72.2 to 72.6 eV. Table III summarizes the effect of surface treatments on the aluminum composition, in atomic percentages, on the samples.

High-resolution Ols spectra showed characteristic broadening of the peak towards the higher binding

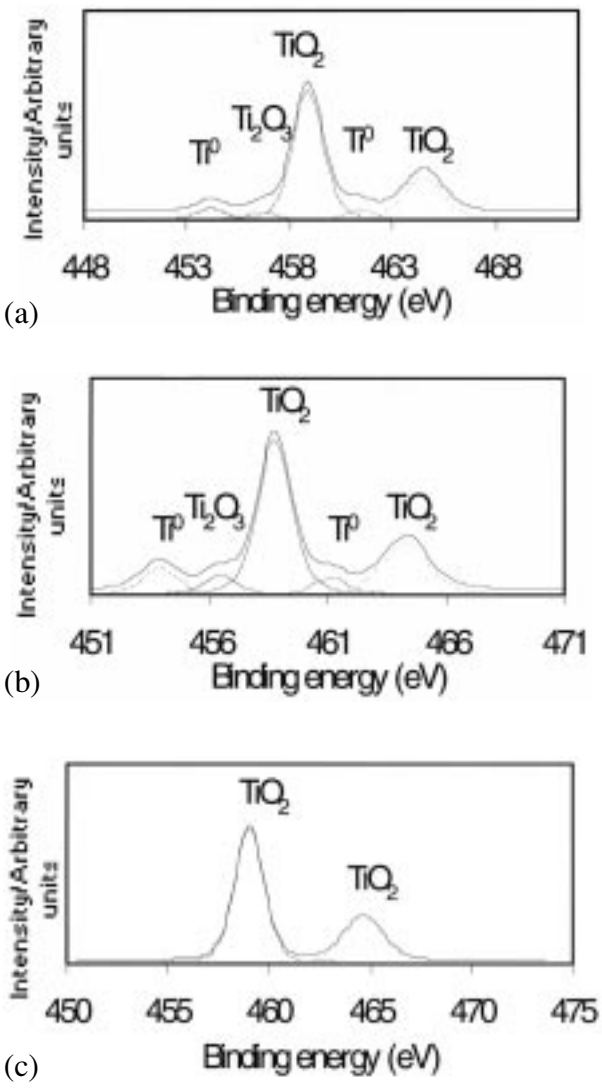

Figure 1 (a) cpTi/Ti64 Ti2p spectra. (b) cpN10/64N10 Ti2p spectra. (c) cp400/64400 Ti2p spectra.

TABLE II Titanium surface composition in atomic percentages for the treated surfaces

Relative at %	Ti^0	Ti_2O_3	TiO_2
cpTi	8.37	3.35	88.28
cpN10	13.35	5.76	80.89
cp400	0.00	0.00	100.00
Ti64	12.71	4.70	82.59
64N10	13.07	5.82	81.11
64400	0.00	0.00	100.00

TABLE III Aluminum surface composition in atomic percentages for Ti64-treated surfaces

Relative at%	Al^0	Al_2O_3
Ti64	6.04	93.96
64N10	0.00	100.00
64400	0.00	100.00

energy side of the major peak, for all test samples (Fig. 3). The Ols peak region is found to consist of three types of oxygen species. The high binding energy peaks are shifted from the primary peak at 530.0–530.6 eV, by approximately 1.5 eV and 3.0 eV. Binding energy changes of this magnitude have been observed on hydrated TiO_2 surfaces [14]. The major peak was attributed to bulk oxygen of TiO_2, the subpeaks at 1.5 eV to the physisorption H_2O and OH(s), and the peak at 3.0 eV to chemisorbed H_2O (TiOH).

The XPS spectra of the surface-treated samples show distinct differences in surface chemistry, related to the amount of Ti^0 and Ti_2O_3 for all samples and Al^0 on the Ti6A14V alloy samples. It is clear that Ti^0 and Ti_2O_3 were not observed on the heat-treated samples, thus indicating that the effect of the heat treatment was to change the Ti^0 and Ti_2O_3, present on the non-heat-treated samples, to TiO_2. The same effect was seen in the alloy samples Al^0 content. In the case of the untreated, as-polished samples, Al^0 was present, but on the heat-treated samples the surface aluminum content was in the form of Al_2O_3. It was not clear why Al^0 was not found on the nitric acid passivated samples. The presence of the acidic and basic OH groups on the treated surface were similar in all cases. It was thought that weakly bonded (physisorbed H_2O) OH groups may have been removed by the heat treatment process [15], but this was not evident on the samples tested in this study.

Though the surface analysis results showed variations in surface groups, it was necessary to investigate biocompatibility and how the surface characteristics, if at all, affected cell activities.

3.2. Cell culture tests

SER + was used, as a control solution, because it contains the necessary nutrients and proteins required for cell growth. SERF investigates the importance of serum proteins in the initial attachment process. PBS supplies the cells with the minimal conditions to keep the cells hydrated. The cellular activity measurements of surfaces in SER +, SERF and in PBS showed that there was no significant difference between the three sample types when compared within the three types of medium separately. Also it was seen that over the 90 min time period the activity of the cells was fairly constant in all cases, Fig. 4 shows an example of AB results for cells seeded on cpTi in SER +. These results indicate that the initial cellular activity does not significantly differ with varying surface chemistry of the samples.

As stated earlier, the literature has shown that similar surface treatments result in marked changes in the dissolution of metal ions from the cpTi and Ti6A14V alloy samples with time. Initial stages of biocompatibility testing may not therefore allow for large variations

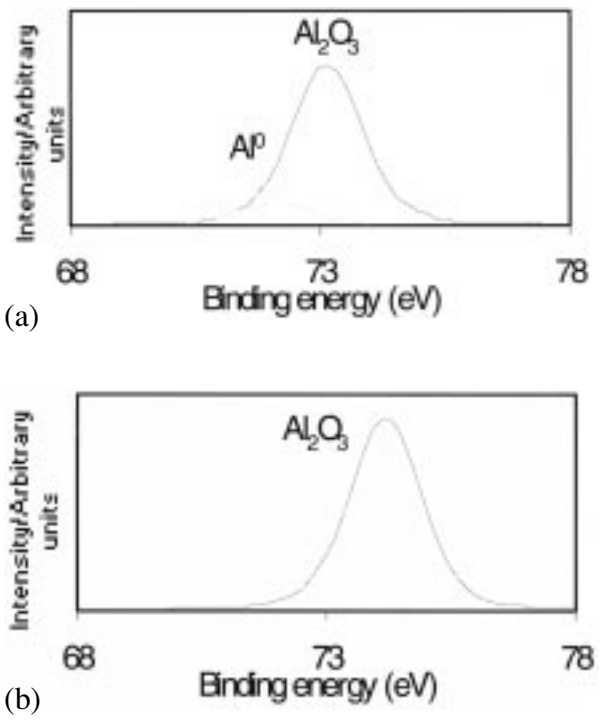

Figure 2 (a) Ti64/A12p spectra. (b) 64400 A12p spectra.

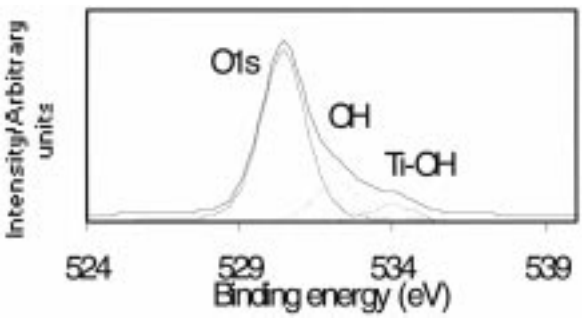

Figure 3 Characteristic high-resolution O1s spectra.

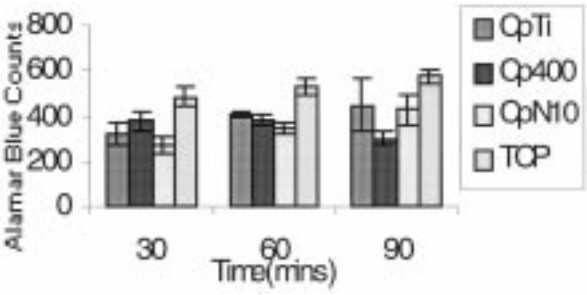

Figure 4 cpTi-treated surfaces; AB results in SER +.

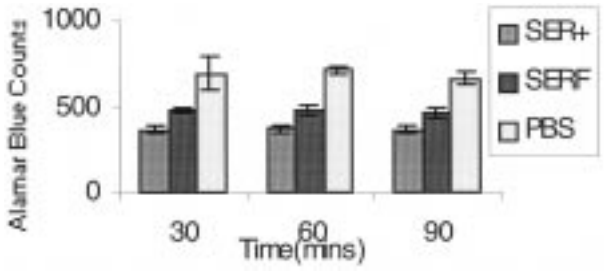

Figure 5 64N10 surfaces; AB result in the three types of media.

in the amount of metal ion dissolution taking place from the various surfaces. Thus, with all surfaces containing TiO_2 as the major component, cellular activity is seen to be similar. It is probable that more obvious variations will be seen over longer time periods when the variations in surface properties become more apparent.

Comparisons of the cpTi and Ti6A14V alloy samples in the three types of media were made. A characteristic graph shown in Fig. 5, shows that for each time point, there was a significant difference in cellular response. All surfaces showed the greatest activity in PBS. SER + and SERF media were similar in AB results (SER + and SERF << PBS).

These preliminary studies only give values of the cell activity but do not give an indication of how the PBS interacts with the surface to produce the significantly higher cell activities. It is known that electrostatic forces between the cells and treated surfaces aid the initial attachment of cells [16]. The sites for electrostatic attraction on titanium and titanium alloy surfaces are thought to be provided by the acidic and basic OH groups on the hydrated oxide. Competition for these sites by media constituents and cells, may be a governing factor of the amount of cells able to attach and hence the activity values shown by the AB assay. PBS contains a reduced number of inorganic salts in solution in comparison to SER + and SERF [17], which also contain a number of amino acids and vitamins. In addition to this SER + contains proteins, all of which will be vying for the same sites of attachment as the cells. The reduced competition in the PBS may result in more of these sites being available to the cells and thus resulting in increased attachment and cell activity over the initial attachment period.

It should also be noted that the high activity of cells in the PBS could be due to cell death taking place. In order to extend these preliminary studies it is necessary to investigate fully the initial attachment of human osteoblast cells in the three types of media. Using cell counts and SEM analysis would give exact numbers for cell attachment as well as indications on whether the cells were showing a healthy morphology.

4. Conclusions

Preliminary results of the surface analysis and cell culture experiments indicate that:

- The surface of cpTi and Ti6A14V alloy changes markedly with heat treatment.
- Changes in surface chemistry seem not to have an effect on cell activity.
- Activity of human osteoblast cells is highest in PBS for all of the treated surfaces.

Acknowledgments

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